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**Screening of Synergistic Interactions of Epigallocatechin-3-gallate with
Antiangiogenic and Antitumor Compounds**

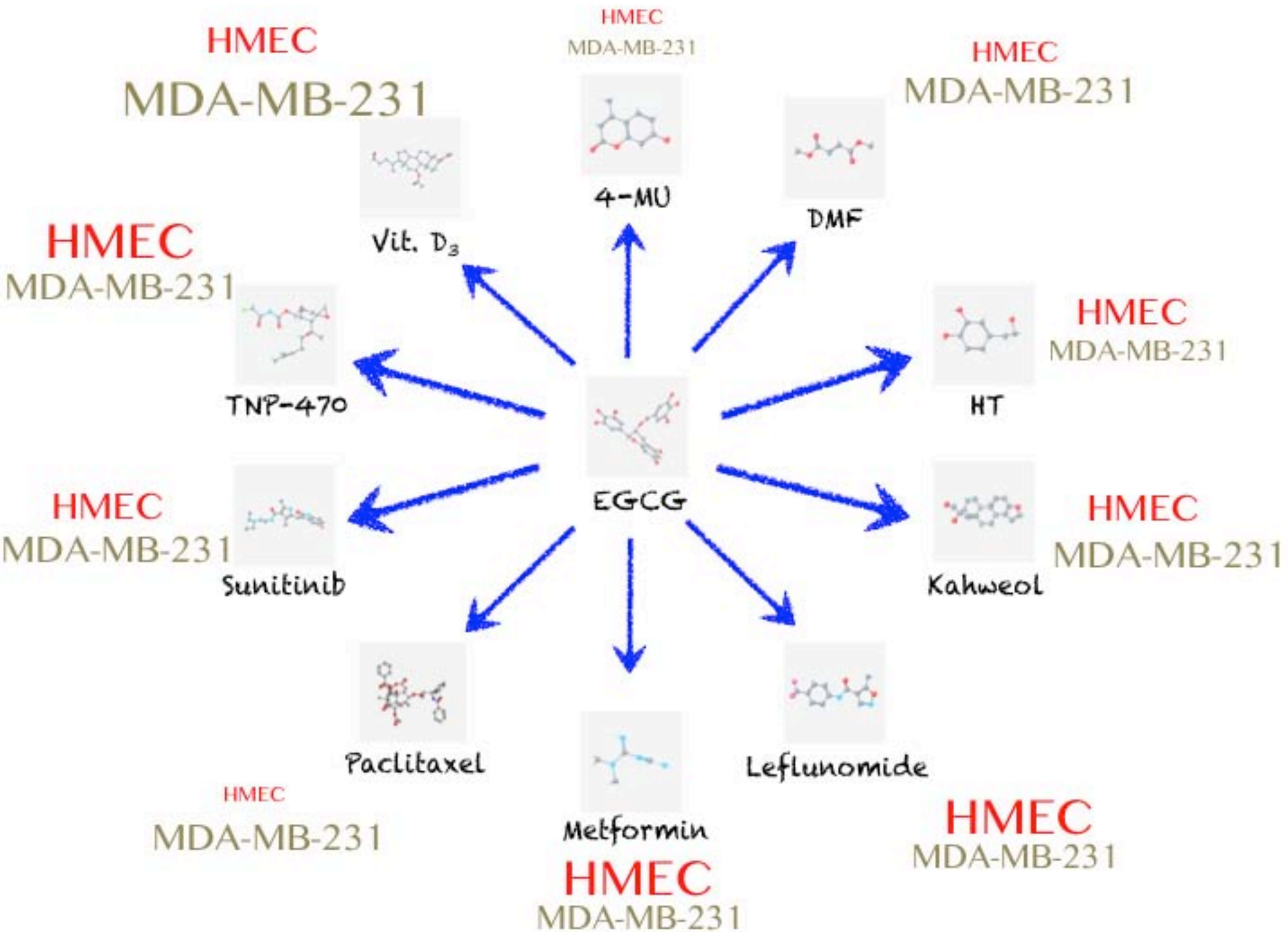
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HIGHLIGHTS

- A screening of combinations with EGCG is carried out with the MTT assay.
- EGCG synergistically interact with 4-methylumbelliferone.
- EGCG is an antagonist of the antiproliferative effects of vitamin D₃.

Summary

Purpose: To screen for possible synergistic interactions of epigallocatechin-3-gallate (EGCG) with a selection of 10 anti-angiogenic or anti-tumor compounds on the survival of endothelial and tumor cells.

Methods: Human HMEC endothelial and MDA-MB231 breast cancer cells were treated with different concentrations of EGCG and the 10 tested compounds either as single agents or in paired combinations with EGCG for 3 days and final survival of cells was determined by the MTT assay. IC₅₀ values, sensitization factors and combination indexes were calculated.

Results; IC₅₀ values of 140±2 and 45±6 µM were determined for EGCG-treated endothelial and tumor cells, respectively. IC₅₀ values for all tested compounds were within the micromolar and the submillimolar range. The values of the sensitization factor increased and those of the combination index decreased for paired combinations of EGCG with 4-methylumbelliferone. The opposite was true for the combination of EGCG with vitamin D₃. Other tested combinations did not exhibit a clear monotonic effect but rather a biphasic behaviour.

Conclusion: Combinations of EGCG and 4-methylumbelliferone synergistically decrease endothelial and tumor cell survival. In contrast, the presence of EGCG antagonizes with the antiproliferative effect exerted by vitamin D₃ on endothelial and tumor cells.

Keywords: EGCG; angiogenesis; cancer; synergy; MTT assay

1. Introduction

Solid cancers have several common characteristics that Hanahan & Weinberg named as the hallmarks of cancer [1]. Angiogenesis is an essential hallmark of cancer because tumor cells need oxygen and nutrients delivered by the vascular system [2]. In fact, tumor growth and metastasis are angiogenesis dependent processes, and microvascular endothelial cells recruited by tumors have become an important target in cancer therapy [1-3].

Drug discovery efforts have identified several potential therapeutic targets in endothelial cells and selective inhibitors capable of slowing tumor growth or producing tumor regression by blocking angiogenesis in *in vivo* tumor models [4]. However, the currently available therapies have limited success in patients, due to complex mechanisms of resistance of the tumor cells [5,6]. One approach to overcoming these problems is to use combinations of drugs with different modes of action that may lead to enhanced antitumor and antiangiogenic effects without injuring the host [5-7]. The combined use of two drugs may sometimes produce enhanced, unchanged or diminished effects in comparison with their individual effects. These three different types of behaviour of the interacting drugs are called synergy, additive/indifferent and antagonistic effects [8].

Our research group is devoted to the screening, identification and characterization of new modulators of angiogenesis. In the last years we have identified a number of natural compounds as new inhibitors of angiogenesis [see for instance 9-14]. We have also claimed for the need of combinatorial approaches to manage angiogenesis [5].

Epigallocatechin-3-gallate (EGCG) is an abundant polyphenol in green tea leaves with a number of biological activities, including antiangiogenesis [5], anticarcinogenesis [16-

18], antimetastasis [19], as well as cancer chemoprevention, among others [20]. Furthermore, our group has previously shown that EGCG is a potent antiinflammatory compound [21,22] targeting histidine decarboxylase [23-25].

In the present work, we analyze paired combinations of EGCG with other 10 bioactive compounds (see Table 1), some described by our research group as potent antiangiogenic compounds, and others currently used in clinical therapy [11-14, 26-32].

2. Material and methods

2.1. Materials

Metformin, leflunomide, TNP-470, sunitinib malate, kahweol, cholecalciferol (vitamin D₃), 4-methylumbelliferone, dimethyl fumarate, Paclitaxel and epigallocatechin-3-gallate were purchased from Sigma Chemicals Co. (St. Louis MO, USA). Hydroxytyrosol was supplied by Extrasynthèse (Lyon, France). Supplements and other chemicals not listed in this section were obtained from Sigma Chemicals Co. (St. Louis MO, USA). Cell culture media, penicillin, streptomycin and amphotericin B were purchased from Biowhittaker (Walkersville, MD, USA). Fetal bovine serum (FBS) and human serum (HS) were products of Harlan-Seralab (Belton, United Kindom). Plastics for cell culture were supplied by NUNC (Roskilde, Denmark) and VWR (West Chester, Pennsylvania, USA).

2.2. Cell culture

The immortalized human microvascular endothelial cell (HMEC) line was kindly supplied by Dr. Arjan W. Griffioen (Maastrich University, The Netherlands). This immortalized cell line has been previously characterized [33]. HMEC cells were grown

in RPMI 1640 medium supplemented with glutamine (2mM), penicillin (50 IU/mL), streptomycin (0.05 mg/mL), and amphotericin (1.25 mg/L) supplemented with 10% fetal bovine serum, and 10% human serum. Human breast cancer carcinoma MDA-MB-231 cells were purchased from ATCC and maintained in RPMI 1640 supplemented with glutamine (2mM), penicillin (50 IU/mL), streptomycin (0.05 mg/mL), and amphotericin (1.25 mg/L) supplemented with 10% fetal bovine serum. Cell cultures were maintained at 37°C under a humidified 5% CO₂ atmosphere.

2.3. MTT cell growth assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay in 96-well microplates was used. The assay is dependent on the reduction of MTT by mitochondrial dehydrogenases of viable cells to a blue formazan product, which can be measured spectrophotometrically. Tumor cells (2.5×10^3 cells in a total volume of 100 μ L of complete medium) were incubated in each well with serial dilutions of single compounds or with paired combinations. After 3 days of incubation in the dark (37°C, 5% CO₂ in a humid atmosphere), 10 μ L of MTT (5mg/mL in PBS) was added to each well, and the plate was incubated for a further 4 h (37°C). The formazan was solved in 150 μ M of 0.04 N HCl-2 propanol, and samples were spectrophotometrically measured at 550 nm. All determinations were carried out in quadruplicate, and at least three independent experiments were carried out. IC₅₀ values were calculated as those concentrations of compound yielding 50% of cell survival, taking the values obtained for control as 100%. Firstly, we calculated IC₅₀ values for each single compound on both cell lines. Once we knew these values for single compounds, we performed the different combinations of EGCG with the 10 selected compounds (Table 1) at concentrations from $4 \times \text{IC}_{50}$ to $1/8 \text{ IC}_{50}$.

From these experiments, the effect of paired combinations was evaluated with two different approaches: determination of combination indexes and sensitization factors (see sections 2.4 and 2.5).

2.4. Combination index (CI)

The so-called combination index (CI) is defined as: $CI = [C_a/IC_a] + [C_b/IC_b]$, where C_a and C_b are the concentrations of compounds A and B used together to achieve a fixed effect. IC_a and IC_b are the concentrations of A and B, respectively required individually to achieve the same effect [8]. Here, we have used as a fixed effect that corresponding to IC_{50} . A CI value of less than, equal to, and more than one, indicates synergy, additivity and antagonism, respectively, between these two compounds.

2.5. Sensitization factors

The sensitization factors indicate the degree of sensitization induced by EGCG on the effects of the 10 selected compounds on cell survival. They were calculated as the ratio of IC_{50} (control cells)/ IC_{50} (EGCG treated cells).

3. Results

3.1. Studies of cell growth and survival with single compounds

Figure 1 shows the survival curves (determined with the MTT assay) of both HMEC endothelial and MDA-MB231 tumor cells treated with EGCG. From these survival curves IC_{50} values of 140 ± 2 and 45 ± 6 μ M were determined for endothelial and tumor cells, respectively. Following the same procedure, the dose-response histograms

(Figures 2 and 3) and IC_{50} values (Tables 2-3) were obtained for both HMEC endothelial and MDA-MB231 tumor cells treated with each of the 10 selected single compounds. All IC_{50} values were within the micromolar and the submillimolar range. For both HMEC endothelial and MDA-MB231 tumor cells, the lowest IC_{50} values are obtained with Paclitaxel (0.7 ± 0.0 and 1.1 ± 0.2 μ M, respectively) and the highest ones with 3-methylumbelliferone (651.8 ± 38.5 and 626.7 ± 204.9 μ M, respectively). No relevant cell specificity was found, with the notable exception of vitamin D₃, a compound that exhibited a much higher toxicity for MDA-MB231 tumor cells ($IC_{50}=33.7 \pm 3.0$ μ M) than for HMEC endothelial cells ($IC_{50}=331.5 \pm 6.2$ μ M).

3.2. Sensitivity of HMEC to the selected compounds in the presence of EGCG

Figure 2 and Table 2 show the effects of exposure schedules of EGCG on HMEC endothelial cells treated with each of the 10 selected test compounds (Table 1). Changes in the IC_{50} (Table 2) values at the different EGCG concentrations tested are displayed along with the corresponding sensitization factor values calculated as described in Material and methods. As observed in this table, the values of the sensitization factor were higher than 1 and they increased with increasing EGCG concentrations in the cases of 4-methylumbelliferone, dimethylfumarate, Paclitaxel and sunitinib. There seemed to be a biphasic behaviour (with sensitization factor values lower than 1 at lower EGCG concentrations and higher than 1 at the highest tested EGCG concentrations) in the cases of hydroxytyrosol, leflunomide, metformin and TNP-470. However, with the exception of leflunomide, the pro-sensitizing effect of EGCG was only observed at 560 μ M EGCG, with values below 1 for the rest of tested EGCG concentrations. Finally, it is noteworthy the very strong anti-sensitizing effect of EGCG in vitamin D₃-treated HMEC endothelial cells.

3.3. Sensitivity of MDA-MB231 breast cancer cells to the selected compounds in the presence of EGCG

Figure 3 and Table 3 show the effects of exposure schedules of EGCG on MDA-MB231 tumor cells treated with each of the 10 selected test compounds (Table 1). Changes in the IC_{50} (Table 3) values at the different EGCG concentrations tested are displayed along with the corresponding sensitization factor values calculated as described in Material and methods. In contrast with the data obtained for HMEC endothelial cells, in the case of MDA-MB231 tumor cells EGCG treatment gave rise to a biphasic behaviour for most of the treatments, with anti-sensitizing effects at lower concentrations and pro-sensitizing effects at higher concentrations of EGCG.

3.4. Combination indexes of EGCG in combination with the 10 selected test compounds

Table 4 shows the CI values obtained for paired combinations of EGCG and each of the 10 selected compounds in both HMEC endothelial and MDA-MB231 tumor cells. Synergistic effects are only observed for the combinations of EGCG with 4-methylumbelliferone and Paclitaxel in HMEC endothelial cells and EGCG with 4-methylumbelliferone in MDA-MB231 tumor cells. For most of the combinations CI values higher than 1 are shown, indicating an antagonistic effect of EGCG on the treatments. The most relevant antagonistic effect seems to be that of the combination of EGCG with vitamin D₃.

4. Discussion

In the last years the convenience of combinatory approaches yielding synergistic antitumoral and antiangiogenic effects are more and more claimed [5,7]. EGCG is a natural compound with a wide array of biological activities. In fact, EGCG has been shown to have antitumor, antiangiogenic and antiinflammatory effects [15-25]. This makes EGCG a potentially interesting candidate for screening studies searching for synergistic combinations.

Some previous studies have analyzed the effects of combinations of EGCG with several compounds. For instance, EGCG nanoparticles enhance the cancer chemosensitization potential of cisplatin [34]. Combinations of EGCG with IL-1R antagonist are effective downregulating IL-1-induced tumorigenic factors in U-2 OS human osteosarcoma cells [35]. Another combination with positive inhibitory effects is that of EGCG combined with capecitabine, which inhibits angiogenesis and tumor growth in nude mice with gastric cancer xenografts [36]. Some combinations of EGCG have been reported to induce antagonistic effects. Thus, EGCG has been shown to inhibit IGF-I-stimulated lung cancer angiogenesis and nicotine-induced migration and invasion of non-small cell lung cancer cells [37,38]. On the other hand, there are some few studies reporting synergistic interactions of EGCG with other compounds. EGCG has been reported to sensitize human prostate cancer cells to TRAIL-mediated apoptosis, both treatments synergistically inhibiting angiogenesis and metastasis associated biomarkers [39]. Another study demonstrated that curcumin synergizes with EGCG when administered in a sequential fashion overcoming stromal protection of chronic lymphocytic leukemia B cells [40]. EGCG and Vorinostat exhibit synergistic anticancer effects against human cholangiocarcinoma cells [41].

For the screening of potential synergistic interactions of EGCG combined with other compounds, we decided to use as a first approach the analysis of the effects of endothelial and tumor cell survival as determined by the MTT assay, a simple, rapid and inexpensive procedure widely used for screening purposes. The list of 10 selected compounds for screening of their paired combinations with EGCG (Table 1) includes 4 natural compounds previously described as antiangiogenic agents by our group, namely, 4-methylumbelliferone [14], dimethyl fumarate [11], hydroxytyrosol [13] and kahweol [12]. The list includes other two antiangiogenic compounds (leflunomide and TNP-470) and four antitumoral compounds (metformin, Paclitaxel, sunitinib and vitamin D₃). Paclitaxel and sunitinib are currently used in the clinic. Paclitaxel is currently used for the treatment of several kinds of cancer, including breast, ovarian and lung cancer [26]. Sunitinib has been described as an antitumoral agent by its potent inhibition of the phosphorylation of tyrosin kinases and was approved in 2006 by FDA as an antiangiogenic therapy [27]. TNP-470 is an antiangiogenic compound with a very strong inhibitory effect on endothelial cell proliferation [28]. Leflunomide is an immunomodulatory drug inhibiting mitochondrial enzyme dihydrorotate dehydrogenase, which plays a key role in the *de novo* synthesis of the pyrimidine ribonucleotide uridine monophosphate (rUMP) [29]. Metformin is a compound used in diabetes treatment and as antitumoral because it interferes with the mTOR pathway [30,31]. The beneficial role of the vitamin D₃ in suppressing inflammation with subsequent colon cancer prevention is well documented [32].

Although in the present study we have made use only of the IC₅₀ values, sensitization factors and combination indexes, other parameters potentially useful can be determined from the experimental data obtained with such a kind of screening. For instance, IC₉₀ values are calculated as those concentrations of compound yielding 10% of cell

survival, taking the values obtained for control as 100%. Taking altogether the set of results shown in Figures 2 and 3 and Tables 2-4, most of the tested combinations do not yield synergistic effects at all. In fact, only the combination of EGCG with 4-methylumbelliferone revealed consistent sensitizations and CI values below 1 pointing to a synergistic effect of both compounds on both endothelial and tumor cell survival. Recent published results point to the potential of 4-methylumbelliferone as an effective chemopreventive and therapeutic agent based on its proven antiangiogenic and antitumor effects [42,43]. In contrast, the strong antagonistic effect of the combination of EGCG with vitamin D₃ is noteworthy. It seems as if the presence of any of these two compounds protects cells from the toxicity of the other compound, leading to an increase of the respective, effective IC₅₀ values. An interesting cell-specific case is that of Paclitaxel, since EGCG synergizes with it in HMEC endothelial cells and antagonizes with it in MDA-MB231 tumor cells.

5. Conclusion

Data presented here show that, in spite of the wide range of biological effects previously demonstrated for EGCG, it does not synergize with most of the ten tested antiangiogenic or antitumor compounds. EGCG produces synergistic effects on both endothelial and tumor cell survival when combined with 4-methylumbelliferone. On the other hand, EGCG behaves as a very strong antagonist of the antiproliferative effects of vitamin D₃. Therefore, these two combinations deserve to be further analyzed in the future with additional pre-clinical assays to test whether these synergistic and antagonistic effects on cell survival are accompanied (or not) by other effects on key steps of angiogenesis and other hallmarks of cancer. For instance, potential synergistic effects on apoptosis could be tested by carrying out cell cycle analysis by flow cytometry,

mitochondrial membrane potential determination, caspase activation assay and poly (ADP-ribose) polymerase cleavage determination by Western blotting, among others.

Conflicts of interest

All authors declare they have no actual or potential competing financial interest.

Authors' contributions

JAGV carried out all the experiments and helped in the managing of bibliography. ARQ interpreted the results and revised the manuscript. MAM conceived the experimental work, interpreted the results and wrote and revised the manuscript. All authors read and approved the final manuscript.

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Figure legends

Figure 1 Dose-response curves showing the effect of EGCG treatment on HMEC endothelial (circles) and MDA-MB231 tumor (squares) cell survival. Data are means \pm SD for three independent experiments (each with 4 replicates of each test point).

Figure 2 HMEC endothelial cell dose-response histograms showing the effect of paired compounds. A) 4-MU/EGCG, B) DMF/EGCG, C) HT/EGCG, D) Kahweol/EGCG, E) Leflunomide/EGCG, F) Metformin/EGCG, G) Paclitaxel/EGCG, H) Sunitinib/EGCG, I) TNP-470/EGCG, J) Vit. D3/EGCG. Data are shown as means \pm S.D. for 3 independent experiments (each with 4 replicates of each test point).

Figure 3 MDA-MB231 tumor cell dose-response histograms showing the effect of paired compounds. A) 4-MU/EGCG, B) DMF/EGCG, C) HT/EGCG, D) Kahweol/EGCG, E) Leflunomide/EGCG, F) Metformin/EGCG, G) Paclitaxel/EGCG, H) Sunitinib/EGCG, I) TNP-470/EGCG, J) Vit. D3/EGCG. Data are shown as means \pm S.D. for 3 independent experiments (each with 4 replicates of each test point).

Tables

This manuscript includes 4 Tables.

Compound	Molecular structure	Describe as...	Molecular target
4-methylumbelliferone (4-MU)		Anti-angiogenic	Inhibitor of hyaluronic acid synthesis
Cholecalciferol (Vit D ₃)		Anti-tumoral	Inhibitor signalling of Wnt pathway
Dimethylfumarate (DMF)		Anti-angiogenic	Inhibitor of VEGFR-2
Epigallocatechin gallate (EGCG)		Anti-angiogenic	Inhibitor of topoisomerases
Hydroxytyrosol (HT)		Anti-angiogenic	Inhibitor of MMP-2 activity
Kahweol		Anti-angiogenic	Inhibitor of MMP-2, uPA activity and VEGF
Leflunomide		Anti-angiogenic	Inhibitor of polymerization of β -tubulin
Metformin		Anti-tumoral	Inhibitor of TOR pathway
Paclitaxel		Anti-tumoral	Inhibitor of the polymerization of β -tubulin
Sunitinib		Anti-tumoral	Inhibitor of tyrosine kinases
TNP-470		Anti-angiogenic	Stimulating the production of cyclin-dependent kinase inhibitor p21 and inactive methionine aminopeptidase-2

Table 1. Names, structures and molecular targets of the compounds tested in this study.

Table 2. Effect of exposure schedule of EGCG on the 10 tested compounds and sensitization on HMEC endothelial cells. IC₅₀ values are given as means±SD of three

	4-MU		DMF		HT		Kahw
EGCG (μM)	IC ₅₀ (μM)	Sensitization factor	IC ₅₀ (μM)	Sensitization factor	IC ₅₀ (μM)	Sensitization factor	IC ₅₀ (μM)
0	626.7 ± 204.9	-	66.0 ± 2.4	-	78.6 ± 8.5	-	37.2 ± 2.9
5.6	876.9 ± 2.9	0.7	88.1 ± 5.8	0.7	208.0 ± 3.9	0.4	> 148.6
11.3	946.9 ± 146.6	0.7	60.3 ± 19.2	1.1	222.0 ± 2.5	0.4	74.4 ± 3.8
22.5	< 78.3	> 8.0	50.5 ± 7.9	1.3	193.1 ± 6.5	0.4	< 4.6
45	< 78.3	> 8.0	10.8 ± 3.6	6.1	10.1 ± 1.6	7.8	< 4.6
90	< 78.3	> 8.0	< 8.3	> 7.0	< 9.8	> 8.0	< 4.6
180	< 78.3	> 8.0	< 8.3	> 7.0	< 9.8	> 8.0	< 4.6

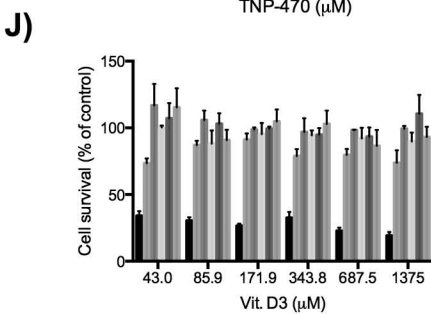
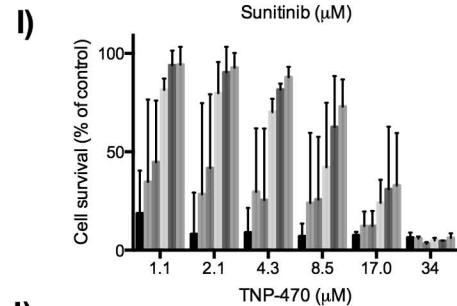
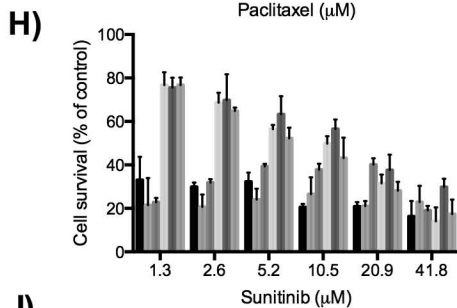
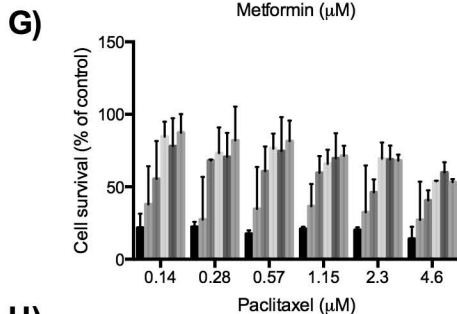
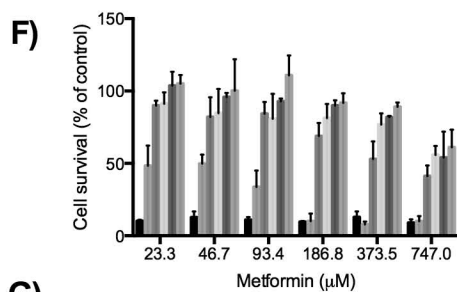
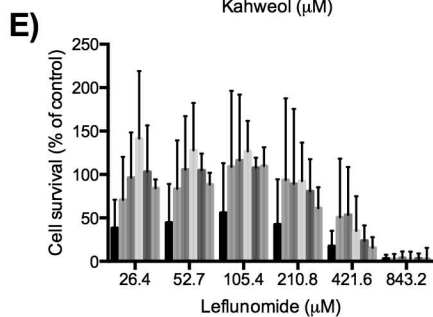
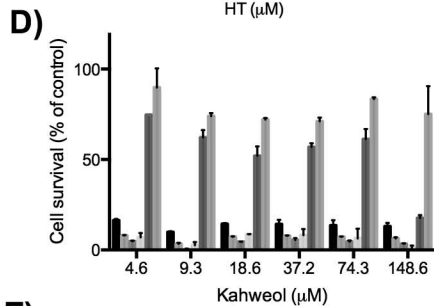
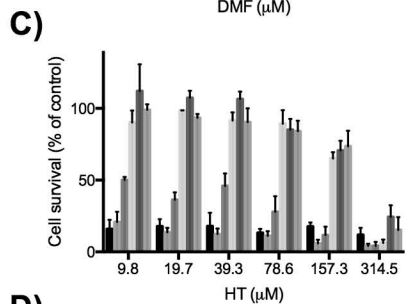
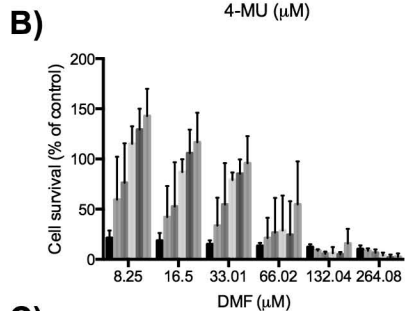
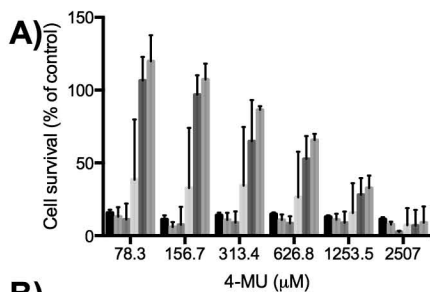
independent experiments.

	Metformin		Paclitaxel		Sunitinib		TNP-4
EGCG (μM)	IC ₅₀ (μM)	Sensitization factor	IC ₅₀ (μM)	Sensitization factor	IC ₅₀ (μM)	Sensitization factor	IC ₅₀ (μM)
0	186.8 ± 8.7	-	1.1 ± 0.2	-	10.5 ± 1.2	-	8.5 ± 1.3
5.6	> 747.0	< 0.2	> 4.6	< 0.2	8.1 ± 2.6	1.3	15.0 ± 7.2
11.3	> 747.0	< 0.2	> 4.6	< 0.2	13.8 ± 4.3	0.8	12.0 ± 3.1
22.5	> 747.0	< 0.2	> 4.6	< 0.2	6.7 ± 0.8	1.5	7.4 ± 3.3
45	> 747.0	< 0.2	2.2 ± 1.9	0.5	< 1.3	> 8.1	< 1.1
90	58.0 ± 3.2	3.2	< 0.1	> 11	< 1.3	> 8.1	< 1.1
180	< 23.3	> 8.0	< 0.1	> 11	< 1.3	> 8.1	< 1.1

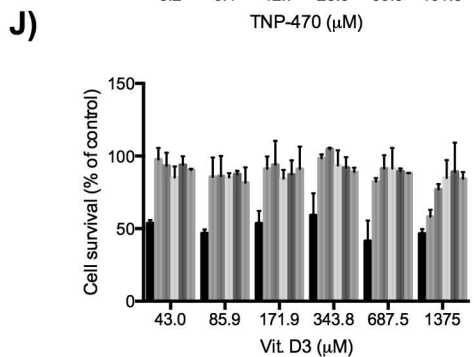
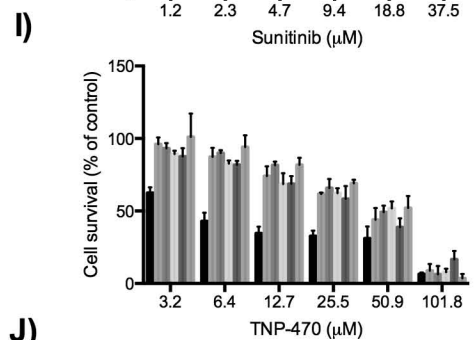
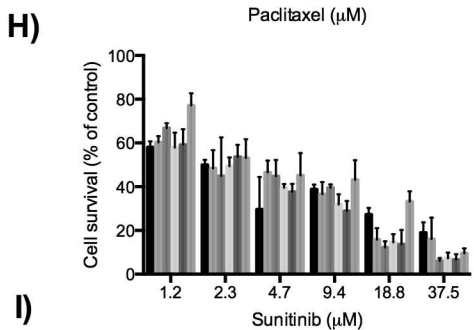
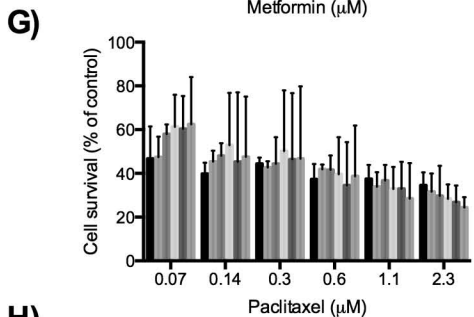
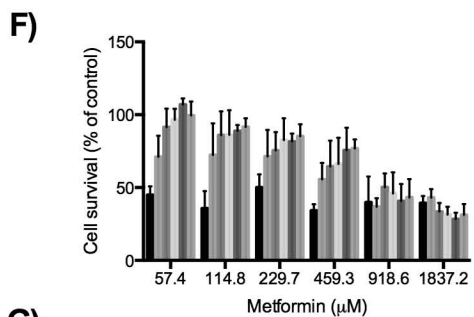
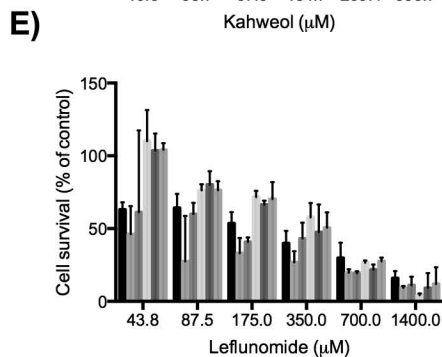
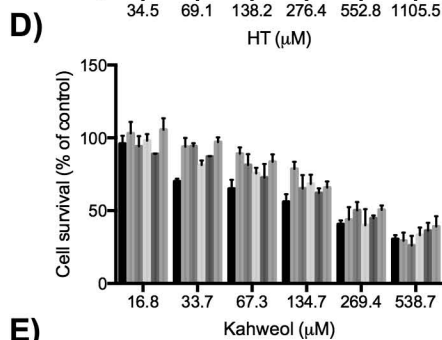
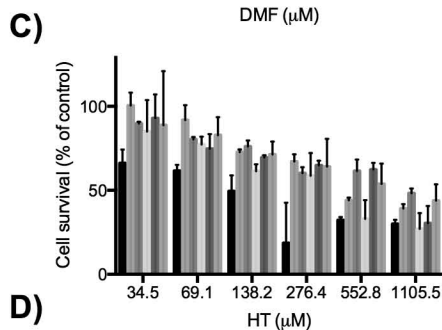
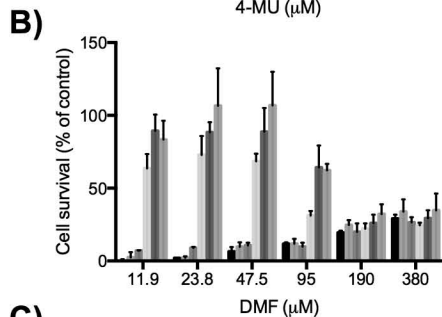
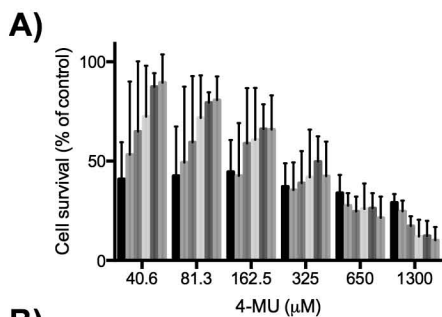
Table 3. Effect of exposure schedule of EGCG on the 10 tested compounds and sensitization on MDA-MB231 tumor cells. IC₅₀ values are given as means±SD of three independent experiments.

Table 4. Combination index (CI) at IC₅₀ of EGCG combined with each of the 10 tested compounds in this study. Mean values are provided.

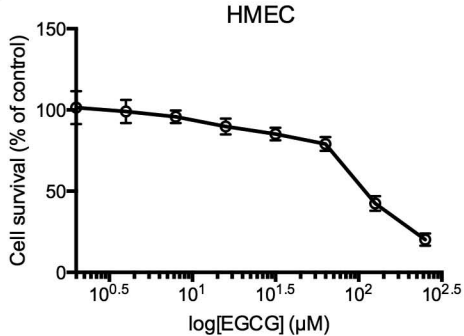
	4-MU	DMF	HT	Kahweol	Leflunomide	Metformin	Placlitaxel	Sunitinib	TNP-470	Vit D3
EGCG (HMEC)	0.75	1.13	2.00	2.71	2.00	3.0	0.77	1.50	3.60	2.30
EGCG (MDA-MB231)	0.88	2.45	1.13	2.13	1.25	1.25	2.55	1.38	1.11	> 4



[EGCG] ■ 180 μM ■ 90 μM ■ 45 μM ■ 22.5 μM ■ 11.3 μM ■ 5.6 μM



[EGCG] 560 μM 280 μM 140 μM 70 μM 35 μM 17.5 μM

A)**B)**